

### Cross- Reference to Related Applications

This application is a continuation of international application number PCT/US99/27505, filed November 19, 1999, which is a continuation-in-part of U.S.S.N. 09/339,632, filed June 24, 1999, now abandoned, which claims the benefit of 60/109,247, filed November 19, 1998.

On page 5, please delete the paragraph at lines 3-25 and replace it with the following paragraph:

While the aforementioned list is a list of biochemical and structural features in a cultured cell-matrix construct formed from dermal fibroblasts, it should be recognized that cultured cell-matrix constructs formed from other types of fibroblasts will produce many of these features and others phenotypic for a tissue type from which they originated. In some cases, fibroblasts can be induced to express non-phenotypic components by either chemical exposure or contact, physical stresses, or by transgenic means. Another preferred embodiment of the invention is a cell-matrix layer having a second layer of cells disposed thereon. The second layer of cells is cultured on the cell-matrix layer to form a bioengineered bilayered tissue construct. In a more preferred embodiment, the cells of the second layer are of epithelial origin. In the most preferred embodiment, the second layer comprises cultured human keratinocytes that together with a first cell-matrix layer, a cell-matrix construct formed from dermal fibroblasts and endogenous matrix to form a dermal layer, comprise a living skin construct. When fully formed, the epidermal layer is a multilayered, stratified, and well-differentiated layer of keratinocytes that exhibit a basal layer, a suprabasal layer, a granular layer and a stratum corneum. The skin construct has a well-developed basement membrane present at the dermal-epidermal junction as exhibited by transmission electron microscopy (TEM). The basement membrane appears thickest around hemidesmosomes, marked by anchoring fibrils that are comprised of type VII collagen, as visualized by TEM. The anchoring fibrils can be seen exiting from the basement membrane and entrapping the collagen fibrils in the dermal layer. These anchoring fibrils, as well as other basement membrane components, are secreted by keratinocytes. It is also known that while keratinocytes are capable of secreting basement membrane components on their own, a recognizable basement membrane will not form in the absence of fibroblasts. Immunohistochemical staining of the skin construct of the present invention has also shown that laminin, a basement membrane protein is present.

On page 7, please delete the paragraph at lines 20-27 and replace it with the following paragraph:

a<sup>2</sup>  
Although human cells are preferred for use in the invention, the cells to be used in the method of the invention are not limited to cells from human sources. Cells from other mammalian species including, but not limited to, equine, canine, porcine, bovine, and ovine sources; or rodent species such as mouse or rat may be used. In addition, cells that are spontaneously, chemically or virally transfected or recombinant cells or genetically engineered cells may also be used in this invention. For those embodiments that incorporate more than one cell type, chimeric mixtures of normal cells from two or more sources; mixtures of normal and genetically modified or transfected cells; or mixtures of cells of two or more species or tissue sources may be used.

On page 10, please delete the paragraph at lines 21-29 and replace it with the following paragraph:

a<sup>3</sup>  
The cultured tissue constructs of the invention do not rely on synthetic or bioresorbable members, such as a mesh member for the formation of the tissue constructs. The mesh member is organized as a woven, a knit, or a felt material. In systems where a mesh member is employed, the cells are cultured on the mesh member and growing on either side and within the interstices of the mesh to envelop and incorporate the mesh within the cultured tissue construct. The final construct formed by methods that incorporate such a mesh rely on it for physical support and for bulk. Examples of cultured tissue constructs that rely on synthetic mesh members are found in U.S. Patent Numbers 5,580,781, 5,443,950, 5,266,480, 5,032,508, 4,963,489 to Naughton, et al.

On pages 16-17, please delete the paragraph at lines 20-30 and 1-6 and replace it with the following paragraph:

ad While not required, the matrix-production medium is optionally supplemented with a neutral polymer. The cell-matrix constructs of the invention may be produced without a neutral polymer, but again not wishing to be bound by theory, its presence in the matrix production medium may assist in collagen processing and deposition more consistently between samples. One preferred neutral polymer is polyethylene glycol (PEG), which has been shown to promote in vitro processing of the soluble precursor procollagen produced by the cultured cells to matrix deposited collagen. Tissue culture grade PEG within the range between about 1000 to about 4000 MW (molecular weight), more preferably between about 3400 to about 3700 MW is preferred in the media of the invention. Preferred PEG concentrations for use in the method may be at concentrations at about 5% w/v or less, preferably about 0.01% w/v to about 0.5% w/v, more preferably between about 0.025% w/v to about 0.2% w/v, most preferably about 0.05% w/v. Other culture grade neutral polymers such dextran, preferably dextran T-40, or polyvinylpyrrolidone (PVP), preferably in the range of 30,000-40,000 MW, may also be used at concentrations at about 5% w/v or less, preferably between about 0.01% w/v to about 0.5% w/v, more preferably between about 0.025% w/v to about 0.2% w/v, most preferably about 0.05% w/v. Other cell culture grade and cell-compatible agents that enhance collagen processing and deposition may be ascertained by the skilled routineer in the art of mammalian cell culture.

On page 17, please delete the paragraph at lines 7-9, and replace it with the following paragraph:

o5 When the matrix producing cells are confluent, and the culture medium is supplemented with components that assist in matrix synthesis, secretion, or organization, the cells are said to be stimulated to form a tissue-construct comprised of cells and matrix synthesized by those cells.

On pages 19-20, please delete the paragraph at lines 26-30 and 1-11 and replace it with the following paragraph:

ab In the method of forming a differentiated epidermal layer, subcultured keratinocytes are taken from the cell stock and their cell numbers are expanded. When a necessary number of cells have been obtained, they are released from the culture substrate, suspended, counted, diluted and then seeded to the top surface of the cell-matrix construct at a density between about  $4.5 \times 10^3$  cells/cm<sup>2</sup> to about  $5.0 \times 10^5$  cells/cm<sup>2</sup>, more preferably between about  $1.0 \times 10^4$  cells/cm<sup>2</sup> to about  $1.0 \times 10^5$  cells/cm<sup>2</sup>, and most preferably at about  $4.5 \times 10^4$  cells/cm<sup>2</sup>. The constructs are then incubated for between about 60 to about 90 minutes at  $37 \pm 1^\circ\text{C}$ , 10% CO<sub>2</sub> to allow the keratinocytes to attach. After the incubation, the constructs are submerged in epidermalization medium. After a sufficient length of time in culture, the keratinocytes proliferate and spread to form a confluent monolayer across the cell-matrix construct. Once confluent, the cell media formulation is changed to differentiation medium to induce cell differentiation. When a multilayer epithelium has formed, cornification media is then used and the culture is brought to the air-liquid interface. For the differentiation and cornification of keratinocytes, the cells are exposed to a dry or low humidity air-liquid interface. A dry or low-humidity interface can be characterized as trying to duplicate the low moisture levels of skin. With time, keratinocytes will express most or all keratins and other features found in native skin when exposed to these conditions.

On pages 20-21, please delete the paragraph at lines 12-30 and 1-11 and replace it with the following paragraph:

Q7  
As mentioned above, the system for the production of a cell-matrix construct may be used in the formation of a corneal construct. The corneal epithelial cells can be derived from a variety of mammalian sources. The preferred epithelial cell is a rabbit or human corneal epithelial cell (corneal keratinocyte) but any mammalian corneal keratinocyte may be used. Other epithelial keratinocytes such as those derived from the sclera (outer white opaque portion) of the eye or epidermis may be substituted, but corneal keratinocytes are preferable. In the method for forming a corneal construct, the medium is removed from the culture insert (containing the cell-matrix construct) and its surround. Normal rabbit corneal epithelial cells are expanded via subculture, trypsinized to remove them from the cultures substrate, suspended in culture medium, and seeded on top of the membrane at a density between about  $7.2 \times 10^4$  to about  $1.4 \times 10^5$  cells/cm<sup>2</sup>. The constructs are then incubated without medium for about four hours at  $37 \pm 1^\circ\text{C}$ , 10% CO<sub>2</sub> to allow the epithelial cells to attach. After incubation, the constructs are submerged in Corneal Maintenance Medium (CMM) (Johnson et al., 1992.) The epithelial cells are cultured until the cell-matrix construct is covered with the epithelial cells. Completeness of epithelial coverage can be ascertained by a variety of methods, for illustration by staining the culture with a solution of Nile Blue sulfate (1:10,000 in phosphate buffered saline). Once the cell-matrix construct is covered, after approximately seven days, the constructs are aseptically transferred to new culturing trays with sufficient corneal maintenance medium (CMM) to achieve a fluid level just to the surface of the construct to maintain a moist interface without submersion of the epithelial layer. The constructs are incubated at  $37 \pm 1^\circ\text{C}$ , 10% CO<sub>2</sub>, and greater than 60% humidity, with the CMM, making media changes, as necessary, typically, three times per week.

On page 21, please delete the paragraph at lines 12-25 and replace it with the following paragraph:

AB In an alternate preferred embodiment, a seeding of a second matrix-producing cell may be performed on a first formed cell-matrix construct to obtain a thicker cell-matrix construct or a bilayer cell-matrix construct. The second seeding can be performed with the same cell type or strain or with a different cell type or strain, depending on the desired result. The second seeding is performed under the same conditions employing the procedures and matrix production medium used in the production of the first layer. One result in performing the second seeding with a different cell type is to have a matrix formed with different matrix component profiles or matrix packing density to affect wound healing when the construct is grafted to a patient. The first cell seeding produces a matrix analogous to the reticular layer of dermis, a more densely packed layer of Type I collagen and constituent extracellular matrix components. The second cell seeding would produce a matrix similar to the papillary layer of dermis characterized by looser collagen fibrils and extracellular matrix. Another result is that the second cell type may produce a therapeutic substance that would also affect wound healing, such as improved graft take or graft integration or the minimization or prevention of scar formation.

On pages 21-22, please delete the paragraph at lines 26-30 and 1-15 and replace it with the following paragraph:

29 In another preferred embodiment, mixed cell populations of two or more cell types may be cultured together during the formation of a cell-matrix construct provided that at least one of the cell types used is capable of synthesizing extracellular matrix. The second cell type may be one needed to perform other tissue functions or to develop particular structural features of the tissue construct. For instance, in the production of a skin construct, dermal papilla cells or epithelial cells from adnexas may be cultured with the matrix-producing cells to allow the formation of epithelial appendages or their components. Epidermal appendages such as sweat or sebaceous gland structures or components or hair follicle structures or components may form when cultured together with the matrix-producing cells. Epithelial cells may be derived from the appendageal structures of gland and hair located in deep dermis, such as by microdissection, and include eccrine cells, myoepithelial cells, glandular secretory cells, hair follicle stem cells. Other cell types normally found in skin that constitute skin may also be added such as melanocytes, Langerhans cells, and Merkel cells. Similarly, vascular endothelial cells may be co-cultured to produce rudimentary components for new vasculature formation. Adipocytes may also be cultured with the matrix-producing cells to form a construct used for reconstructive surgery. As alternate mode of delivery of this second cell type, the cells may be locally seeded as a spot or as an arrangement of any number of spots of cells on or within a forming or completely formed cell-tissue matrix for localized development of these structures. To seed the cells within the cell-matrix construct, the cells may be injected between the top and bottom surfaces, within the cell-matrix, for the cells to grow, form specialized structures and perform their specialized function.

On page 35, please delete the paragraph at lines 6-22 and replace it with the following paragraph:

Cell-matrix constructs were prepared according to the methods in Example 1 using human dermal fibroblasts derived from neonate foreskin and were grafted onto full excision wounds created on nude athymic mice. Mice were grafted according to the methods described by Parenteau, et al. (1996), the disclosure of which is incorporated herein. Grafts were examined at 14, 28 and 56 days for signs of adherence to the wound bed, evidence of wound contraction, areas of graft loss, and presence of vascularization (color). The graft areas were photographed while intact on the mice. A number of mice were sacrificed at each timepoint, and the graft areas and their surrounds were excised along with a surrounding rim of murine skin to at least the panniculus carnosus. Junctions between the graft and the murine skin were preserved in each sample. The explanted tissue samples were then fixed in phosphate buffered 10% formalin and fixation in methanol. Formalin fixed samples were processed for H&E staining according to procedure described in Example 1. Grafts were able to integrate with the mouse skin, with minimal contraction noted. Within 14 days of grafting, the mouse epidermis had migrated completely over the graft. Using the H&E stained samples, vessels were obvious within the graft at 14 days, and throughout the experiment. By gross observation and by H&E stained samples, it was determined that the graft persisted and remained healthy looking (contained living cells, no gross matrix abnormalities, etc.) throughout the length of the experiment.

On page 45, please delete the paragraph at lines 14-28 and replace it with the following paragraph:

all  
Samples were formalin fixed and processed for hemotoxylin and eosin staining for light microscope analysis. Visual histological evaluation demonstrated that the Condition 2 lacking PEG demonstrated a comparably similar matrix as Condition 1 containing PEG. Biochemical analysis measuring the collagen content of the construct showed nearly the same amount of collagen in both:  $168.7 \pm 7.98 \mu\text{g}/\text{cm}^2$  for Condition 1 with PEG as compared to  $170.88 \pm 9.07 \mu\text{g}/\text{cm}^2$  for Condition 2 without PEG. Condition 3 containing high levels of insulin and hydrocortisone showed a higher expression of matrix, including collagen, at a timepoint earlier than the other two conditions. Besides endogenously produced fibrillar collagen, decorin and glycosaminoglycan were also present in the cell-matrix constructs in all Conditions. The cultured dermal construct formed by the method of Condition 2 of this Example is shown in Figure 2. Shown in Figure 2 is a photomicrograph of a fixed, paraffin embedded, hematoxylin and eosin stained section of a cell-matrix construct formed from cultured human dermal fibroblasts in chemically defined medium at 21 days. The porous membrane appears as a thin translucent band below the construct and it can be seen that the cells grow on the surface of the membrane and do not envelope or integrate the membrane with matrix.

On page 46, please delete the paragraph at lines 12-15 and replace it with the following paragraph:

all<sup>2</sup>  
Using a 21 day dermal construct formed by human dermal fibroblasts under chemically defined conditions according to the method of Condition 2 (without PEG) described in Example 15, above, normal human neonatal foreskin epidermal keratinocytes were seeded on the top surface of the cell-matrix construct to form the epidermal layer of the skin construct.